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AUTOIMMUNITY IN AIDS

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<p>Infection with Human Immunodeficiency Virus type 1 (HIV-1), the etiologic agent of the acquired immune deficiency syndrome (AIDS), is increasing at an epidemic rate. The severity of immunologic impairment of infected individuals spans a broad range. It is not currently known whether this heterogeneity reflects distinct outcomes of infection or whether infection progress to a common endpoint at different rates. Developing closer knowledge of immunological events that precede the onset of frank AIDS, as well as the interplay between HIV, immunity and the hematopoietic elements that give rise to the immune system will assist in our understanding of the pathophysiology of AIDS. Such knowledge is also guiding our attempts to develop better therapeutic approaches to AIDS. This proposal focuses on two primary areas reflecting the respective strengths of the two participating laboratories. From their inception these efforts have been highly collaborative. The first section, Interaction of HIV-1 and Bone Marrow, focuses on hematopoietic progenitor cells as possible reservoirs of virus. It is designed to explore the relationship between acquisition of lineage specific differentiated function and viral permissiveness, and examine the interaction of infected myeloid and lymphoid cells as they affect immune function and regulation of bone marrow growth. These studies also bear directly on the use of bone marrow transplantation as a new therapeutic approach to the treatment of HIV associated malignancies and, potentially, AIDS itself. Our project has</p> <p>(Continued on next page)</p>					
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monitored immune reconstitution in the first HIV patients to receive allogeneic bone marrow transplants plus antiviral therapy following full myeloablation. Our second research goal has been the Evaluation of Immunologic Changes Related to Progression of HIV Infection. This aspect of our research draws on the resources of two large prospective cohort studies of the natural history of HIV infection, one in gay/bisexual men (SHARE) and one in intravenous drug users (ALIVE). These studies focus on evaluating changes in phenotypically and functionally defined T cell subpopulations which may represent host responses to HIV infection or may predict decline in immune competence as a result of HIV infection. Specifically, studies to date have examined changes in numbers of double negative (CD4⁺, CD8⁻) T cells, natural killer cells, and in vivo activated IL-2 responsive T cells as a function of disease stage.

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X For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

X In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

Robert J. D. 9/8/89
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V. INTRODUCTION

A: Interaction of HIV-1 and Bone Marrow. The severity of immunohematologic impairment of HIV-infected individuals spans a broad range, culminating in a clinical syndrome characterized by hematologic abnormalities and the profound impairment of immune function. Although immunosuppression can be explained in part by the selective depletion of permissive CD4 positive helper/inducer T lymphocytes, the mechanisms by which the virus interferes with the generation of other marrow derived lineages are not known. In addition to lymphopenia, AIDS and AIDS-related complex (ARC) patients often suffer from a variety of peripheral blood abnormalities including granulocytopenia, thrombocytopenia (Spivak et al., 1984, Treacy et al., 1986), and anemia (Castella et al., 1985). Despite this finding, bone marrow from these subjects ranges from normocellular to hypercellular, with distinct abnormalities in the maturation of all lineages similar to those seen in myelodysplastic syndrome (Schneider and Picker, 1985). Similarly, there is no apparent deficit in multipotential hematopoietic progenitor cells as reflected in normal numbers of bone marrow cells giving rise to erythroid and granulocyte-macrophage colonies in vitro (Donahue et al., 1987). Thus it appears probable that HIV-mediated myelosuppression occurs by interruption of maturation, either by direct noncytolytic infection hematopoietic cells, or by interfering with ancillary cell populations that regulate their growth. Studies of in vitro hematopoietic colony growth implicate T lymphocytes in the regulation of neutrophil, macrophage, eosinophil (Chickkappa and Phillips, 1984), and erythroid (Sharkis et al., 1978) lineages. Thus it is conceivable that marrow dysplasia occurs, at least in part, secondary to the loss or alteration of lymphocyte mediated regulation. However, HIV may be capable of infecting myeloid precursors in vivo (Bush et al., 1986) as well as in vitro (Donahue et al., 1987), leaving open the possibility that while not directly cytotoxic to marrow progenitors, HIV may alter the maturational pathway of infected progenitors or affect their ability to receive and/or respond to regulatory signals.

Of equal importance in terms of viral pathogenesis, immature myeloid cells may serve as a reservoir for replicating or latent virus. Myeloid derived cell lines such as U937, normally highly permissive for virus growth, may be rendered nonpermissive by culture in the presence of granulocyte-monocyte colony stimulating factor (GM-CSF) (Hammer et al., 1986).

Understanding the interactions between HIV and hematopoietic cells is also of potential importance to the rational development of therapeutic strategies for the treatment of AIDS and AIDS related malignancies. Bone marrow transplantation (BMT) has been proposed as a possible treatment for AIDS because it presents the opportunity to reconstitute the immune system with cells derived from a healthy donor. The infectious nature of AIDS, however, has justifiably given rise to the concern that the newly developing immune cells would become infected and suffer the same fate as the recipients own cells. This in fact appears to be the case in a limited number of marrow transfusions performed without the benefit of a marrow ablative preparative regimen (Lane and Fauci, 1985). This problem has prevented the initiation of full scale clinical trials of BMT for the treatment of AIDS. However, the development of antiviral agents such as the analog 3'-Azido-3'-deoxythymidine (AZT, BW A509U, Burroughs Wellcome), raises new possibilities for BMT. It is therefore important to determine how anti-retroviral agents, which themselves may be myelosuppressive, affect the balance of lymphocyte-marrow-HIV interactions.

That the failure of previous transplant attempts may be related to the quantitative extent of virus "burden" at the time of transplant is suggested by our study of BMT recipients who were inadvertently infected with transfusion borne HIV shortly after receiving allogeneic marrow transplants (Duff et al., 1985, Donnenberg et al., submitted for publication). In two well documented cases, HIV infection at a time when the patients were severely immunocompromised resulted in no detectable alteration of the pace or extent of reconstitution. Both patients are alive and leading active lives almost 6 years later, with persistent antibody titers and lymphadenopathy. Although the eventual outcome of infection may be no different in these patients than in individuals infected in health, these results give us reason to hope that under the coverage of appropriate antiviral agents, and with an appropriate marrow ablative regimen, a durable improvement of immunity could be effected in AIDS patients. We are now transplanting HIV seropositive patients presenting with hematologic malignancy in the absence of opportunistic infection (see Holland et al., appended preprint). Preliminary results indicate not only the possibility of significant immune reconstitution, but of reduction of virus load to an undetectable level as well.

B. Evaluation of immunologic changes related to progression of HIV infection. Many immune functions decline as HIV infection progresses (Margolick and Fauci, 1987), and the culmination of this general decline is the onset of opportunistic infections and neoplasms. Key questions that remain

unanswered are at what point does this decline become important for the health of the infected person, and which functions are critical to this adverse health effect? Answers to these questions are necessary for identifying individuals at high risk for progression of HIV infection and for developing effective therapies for HIV infection.

We are approaching these issues in several ways. We hypothesize that a more detailed understanding of the pathogenesis of immune compromise will help us determine which HIV-infected individuals have stable infections and which will progress. We are particularly interested in limiting dilution analysis (LDA) because, as detailed below, LDA can be used to measure the frequency of cells belonging to functionally defined lymphocyte subsets, and yields quantitative information at the level of the single precursor.

Of particular interest is the ability of T lymphocytes to respond to IL-2, a lymphokine which is critical for proliferation of T cells; we and others have hypothesized that defects in the ability of T lymphocytes to proliferate in response to IL-2 might contribute to the loss of cell mediated immunity that is the hallmark of AIDS. Therefore, we have conducted preliminary studies of this basic T cell function in patients at different stages of HIV infection. The results of these studies strongly support the need for studies on patients over time (longitudinal studies) as well as more basic biochemical studies to determine the mechanism or mechanisms responsible for decreased IL-2 responsiveness of cells from patients with advanced HIV infection.

Another approach to identifying individuals with different responses to HIV infection is to analyze the immune response to the infection itself. Changes in T lymphocyte subsets in the peripheral circulation after infection with HIV are well documented and include a decrease in CD4⁺ T cells and an increase in CD8⁺ T cells. We have recently focused attention on other, less well studied lymphocyte populations. We recently demonstrated that when CD3⁺, CD4⁺, and CD8⁺ lymphocytes are determined independently (one-parameter analyses), an increase in the difference between the number of CD3⁺ lymphocytes and the sum of CD4⁺ and CD8⁺ lymphocytes [i.e., $CD3 - (CD4 + CD8)$] occurs at the time of seroconversion and continues afterward (Margolick et al, 1989). This increase is present in both absolute and relative terms and cannot be accounted for by changes in the CD4⁺ and CD8⁺ populations of T cells. It is not known whether this response to HIV infection, or its underlying cause, has prognostic significance, but this possibility merits investigation.

Finally, in view of the critical nature of loss of antigen-induced immune responses in the pathogenesis of AIDS (Lane et al, 1985), we are studying the expression of Class II histocompatibility molecules expressed on antigen-presenting cells (monocytes) from HIV-infected individuals. These molecules (HLA-DR) are essential for normal presentation of antigen to T helper lymphocytes in the initiation of antigen-specific immune responses. Early reports indicated that expression of these molecules by antigen-presenting cells might be diminished in patients with AIDS, suggesting a possible mechanism for impaired cellular immunity in AIDS (Belisto et al, 1984; Heagy et al, 1984). However, more recent reports have not confirmed the early reports (Sei et al, 1986). All of these studies are limited by their cross-sectional design. Because the same patients were not studied over time, the possibility exists that small declines could occur (within the ostensible normal range). We are therefore prospectively evaluating the extent of HLA-DR expression by monocytes from HIV-infected individuals.

VI. BODY

A. Methods

1. Patient populations

a. **SHARE patients.** SHARE participants are gay/bisexual men from the Baltimore-Washington area who had not been diagnosed as having AIDS prior to entry into the study (April - November, 1984). At each semi-annual visit participants undergo interview, physical examination, and laboratory testing. Tests include HIV-1 serology (ELISA and immunoblot for confirmation), complete blood count, screening for gonorrhea and syphilis, and T-cell subset analysis (CD3, CD4 and CD8). Several aliquots of plasma, serum, lymphocytes, semen, urine, and mouthwash are obtained for frozen storage at each semi-annual visit. We also have data on serum immunoglobulins, cytomegalovirus serology, hepatitis-B serology, and routine serum chemistries from the first two visits. Volunteers have been sought from the SHARE cohort for the proposed additional prospective studies.

As of February, 1989, SHARE has completed 10 full cycles of follow-up visits. Follow-up rates have been notably high. More than eighty-five percent of the initial cohort are still being followed. There have been 113 cases of AIDS diagnosed thus far in SHARE; 80 participants who were seronegative at baseline have seroconverted.

b. **Moore Clinic Outpatients.** Patients, staged according to the CDC criteria, were seen at the Moore Clinic of the Johns Hopkins Hospital. Peripheral blood was drawn during routine office visits. Control subjects were Johns Hopkins students and employees who denied belonging to HIV risk groups. Informed consent was obtained according to a protocol approved by the Johns Hopkins Joint Committee for Clinical Investigation.

c. **ALIVE study patients.** AIDS Link to IntraVenous Experiences (ALIVE) is a prospective cohort study of 800 intravenous drug users (IVDUs) which was initiated at Johns Hopkins University in February, 1988. The goal of the study is to determine the natural history of HIV infection in this high risk population. The study was designed to enroll 640 HIV seropositive IVDUs as well as 160 seronegative IVDUs to serve as controls and to prevent stigmatization of study participants. Participants are seen in clinic at 6 month intervals for physical and laboratory examination, including measurement of T cell subsets, as described above for SHARE.

2. **Virus strains.** Low passage monocyctotropic strains SF162 and KW were obtained from, J. Levy and H. Farzadegan, respectively. Strains CD and TD were clinical isolates obtained from H. Farzadegan. HTLV-IIIb was originally obtained from the laboratory of R. Gallo.

3. **Antigens and Mitogens.** Tetanus toxoid, preservative free refined concentrate, the generous gift of Wyeth Laboratories (Marietta, PA.), was used at a concentration of 1 to 5 ug/ml. CMV antigen (MA Bioproducts, Walkersville, MD) was used at a 1/1000 dilution of stock. PHA, CONA and PWM (Sigma Chemicals) were used at 2, 5, and 5 ug/ml, respectively.

4. **Lymphoproliferative Responses.** Peripheral blood mononuclear cells (PBML) from HIV-1 positive subjects or healthy volunteers were resuspended to 7.5×10^5 /ml in complete medium (CM) consisting of RPMI 1640, 10% heat inactivated human AB serum, 2 mM L-glutamine, 10 mM HEPES buffer, 50 ug/ml gentamicin, 100 U/ml penicillin, and 5×10^{-5} M 2-mercaptoethanol. The cells were aliquoted (200 ul/well) in U-bottom microtiter plates (Flow Laboratories, Cat. No. 76-013-05). Antigens were added at $10 \times$ final concentration (see Antigens, above) in triplicate and the plates incubated at 37°C , 5% CO_2 . Cells were cultured with and without the addition of exogenous Interleukin-2 (IL-2, 4 units/ml) on day 3. Serum-free supernatant, obtained from MLA-144 cell culture was used as a source of IL-2. On day 6, the cultures were pulsed with $^3\text{HTdR}$ (0.5 uCi/well) and incubated an additional 4 h before being harvested onto glass fiber filters (Whatman, GF/A) with a multiple automatic sample harvester. $^3\text{HTdR}$ uptake was measured by liquid scintillation spectrometry. Results were expressed as net counts per minute above background (NET CPM), where background counts were determined by culturing in the presence of diluent alone. Analysis was performed using the logarithm of the NET CPM because CPM data are log-normally distributed.

5. **Teflon culture of human bone marrow.** Cells were suspended to 1×10^6 /ml in growth medium consisting of alpha-MEM, 30% FCS, 1% BSA plus antibiotics and cultured in 50 ml Teflon Erlenmeyer flasks (Nalgene), 5 ml/flask. Cultures were supplemented with either lymphocyte conditioned medium (10%) or recombinant human growth factors (rhM-CSF, 1/4,500 dilution of stock plus rhGM-CSF, 5 U/ml, Genetics Institute, Cambridge, MA).

6. **Methylcellulose culture of human bone marrow.** Bone marrow was grown in methylcellulose medium according to a modification (Rowley et al., 1987) of the method of Ogawa et al. (1976). Cells were plated in alpha MEM medium containing 0.8% methylcellulose, 30% fetal calf serum, 1% BSA, 0.1 mM 2-mercaptoethanol and either 10% lymphocyte conditioned medium or recombinant human growth factors.

7. **Preparation of a progenitor enriched bone marrow fraction by elutriation (positive selection).** Mononuclear cells (prepared by Ficoll-Hypaque gradient centrifugation) from heparinized

aspirated bone marrow from HIV⁺ or normal donors were elutriated (3000 rpm, standard chamber, JE6 rotor). 100 ml fractions were collected at flow rates of 17 ml/min (load), 24 ml/min (predominately lymphocytes), and 28 ml/min (monocyte enriched). Cells retained in the chamber at 28 ml/min were flushed by turning off the rotor while maintaining medium flow (R/O fraction, progenitor enriched). Cells from the R/O fraction were resuspended ($80-100 \times 10^6$ cells) in 2 ml Percoll (sg = 1.080) and layered beneath 5 ml of 1.074 Percoll which in turn was layered beneath 1 ml RPMI-1640 medium. Gradients were centrifuged at 1475 rpm for 20 min. The blast cell enriched, lymphocyte and monocyte depleted second band was collected and washed 2x with PBS.

8. Preparation of a progenitor enriched bone marrow fraction (positive selection).

Positive selection of CD34⁺ cells was accomplished by immune adherence. A 60 x 15 mm plastic petri dish was flooded with 3 ml goat anti-mouse IgG (10 ug/ml) in 0.05 M Tris buffer (pH 9.5). After 2 h at room temperature the dish was washed 4 x with PBS-A, 0.2% BSA. Bone marrow mononuclear cells (12×10^6 prepared by Ficoll/Hypaque gradient separation) were incubated for 30 min on ice with anti-MY-10 antibody (20 uL/ 1×10^6 cells), and washed twice with cold PBS-A, 0.2% BSA were centrifuged onto the prepared petri dish (200 RPM for 2 min). The petri dish was then incubated for 30 min at 4°C and subjected to 2 cycles of gentle rinsing (PBS-A, 0.2% BSA) to obtain nonadherent cells, followed by 2 cycles of vigorous rinsing to obtain adherent (positively selected) cells. Phenotype was confirmed by flow cytometry after overnight incubation in growth medium.

9. p24 antigen capture assay. HIV-1 antigen (p24) was detected in culture supernatants

using an enzyme-linked immunosorbant (ELISA) antigen capture assay. The capture reagent, a high titer human serum, diluted 1/100,000 (in carbonate buffer pH 9.6) was adsorbed to microtiter plates (75 ul/well) (FLOW Laboratories, McLean, VA, Cat. no. 76-381-04) overnight at 4°C. The plates were washed 2 times in PBS-TWEEN (0.05%) using a Skatron Micro II plate washer. PBS-A containing 3% bovine serum albumin (BSA) was added to each well (100 uL/well) and incubated 1 h at 37°C. The plates were washed as before. Test supernatants and a positive control standard (human recombinant p24, Du Pont Laboratories) were serially diluted in the plates (75 ul/well, 8 two-fold steps) in PBS-A, 1% BSA, 0.5% NP-40. The starting antigen concentration was 5000 pg for the p24 standard and 1/2 for the test supernatants. The plates were incubated overnight at room temperature and washed as before. Rabbit anti-HIV-1 p24 was added (1/3000, 100 uL/well, MicroGeneSys) and the plates were incubated 4 h at 37°C. After 2 washes, goat anti-rabbit IgG alkaline phosphatase conjugate (SIGMA), diluted 1/300 in PBS-NP40 (0.5%) (1% BSA), was added (100 ul/well) and incubated 2 h at 37°C. The plates were washed and p-nitrophenylphosphate substrate (SIGMA) at 1 mg/ml in diethanolamine buffer (pH 9.8) was added (100 ul/well) and incubated 30 minutes in the dark at room temperature. 50 uL of 3M sodium hydroxide was added to each well and the plates read at 405 nm on a Titertek Multiskan Plate Reader. Antigen concentration was determined relative to the recombinant HIV-1 p24 standard. The assay was directly compared to the licensed test provided by Abbott Laboratories and had similar sensitivity (approximately 50 pg/ml).

10. Two color flow cytometry. Cell surface phenotype was determined by 2 color

immunofluorescence modified from the protocol of Loken et al. (1987) using a Becton-Dickinson FACSCAN analyzer. Briefly, indirect staining was first performed using fluorescein isothiocyanate labeled anti-mouse Ig (affinity purified, F(ab')₂ raised in sheep, Sigma Chemicals). Staining with a second, phycoerythrin conjugated, monoclonal antibody was performed after washing the cells in filter sterilized medium containing PBS-A, 1% normal mouse serum, 4% newborn calf serum, 2% normal goat serum and 2% human AB serum. Monoclonal antibodies were obtained from Ortho Pharmaceuticals (Raritan NJ), Becton-Dickinson Corporation (Mountainview, CA.) and Coulter Immunology (Hialeah, FL). Specimens from SHARE or ALIVE were stained with directly conjugated antibodies as listed under Results using previously described methods (Margolick et al, 1989). Briefly, antibodies were added to 100 ul whole blood for 30 min, the red cells were lysed with a hypotonic ammonium chloride solution, and the cells were washed, fixed in 1% paraformaldehyde, and analyzed on a Coulter EPICS C flow cytometer.

11. Assessment of antigen presentation. Development of immunocompetent antigen

presenting cells from monocyte depleted bone marrow was evaluated after 7 and 14 days culture in the presence of recombinant human colony stimulating factors as described above (5. Teflon culture). Cultured cells (20,000 cells/well) were allowed to adhere to flat bottom microtiter plates for 2 h at 37°C after which

nonadherent cells were washed away with PBS-A. The remaining adherent cells were pulsed with antigen (2 ug/ml tetanus toxoid) and incubated for 2 h at 37°C. The cells were then washed to remove excess antigen, irradiated at 1500 rad and cocultured with 1×10^5 monocyte depleted autologous lymphocytes. The ability of these antigen-pulsed adherent cells to trigger proliferation of T cells was then determined by ^3H thymidine uptake as described above.

12. Limiting dilution analysis of lymphoproliferative response to Interleukin-2. LDA of lymphocyte proliferation was used to estimate the proportion of activated (i.e. IL-2 receptor bearing) circulating T lymphocytes. Assays were performed in U-bottom 96 well plates (Linbro). PBML obtained from Ficoll-Hypaque separated peripheral blood were serially diluted in CM. For experimental wells the medium was supplemented with 25% MLA-144 supernatant (approximately 20 units/ml of IL 2 final concentration). Twelve replicates were plated per dilution. In subsequent experiments, we used 8 four-fold dilutions ranging from 80,000 to 4.9 cells/well. Control cultures grown in the absence of IL-2 were plated in sextuplicate at each cell dilution. Cultures were incubated at 37°C, 5% CO_2 humid atmosphere. On day 6 each well received 0.5 uCi of methyl-tritiated thymidine and was harvested 4 h later. Thymidine uptake was measured by liquid scintillation spectrometry as described above. Experimental wells were scored as positive or negative by comparison to CPM incorporated in control (i.e. without IL-2) cultures assayed at the same cell concentration. A well was considered positive if it exceeded the greater of the geometric mean control CPM plus three standard deviations or 500 CPM.

The frequency of IL-2 responsive cells was estimated by a two-step procedure. Briefly, a preliminary estimate was made by determining the slope of the least squares line of best fit of the log fraction negative wells versus the responder cell concentration. The frequency was then determined by the maximum likelihood approach using the least squares estimate as an initial value. Net counts per minute per precursor (Net CPMP) was calculated at each cell concentration by dividing the net counts per minute (Net CPM) in each positive well by the average number of precursors per positive well. Net CPM was obtained by subtracting background CPM from experimental CPM, where background CPM represents an N weighted mean of control CPM (N=6) and CPM obtained in negative experimental wells. Precursors per positive well was calculated from the LDA frequency estimate and approaches unity at limiting dilution.

13. Limiting dilution analysis of bone marrow progenitor growth. Progenitor enriched bone marrow cells (described above) from healthy subjects (24 h after infection with HIV-1 or Mock infection) or from HIV-1 positive subjects were serially diluted in growth medium (see Teflon cultures) and plated in round bottom wells (200 uL/well). Dilutions were made in half-log₁₀ steps ranging from 300 to 1 cell/well, with one full plate (96 replicates) for each cell concentration. Cultures were evaluated microscopically for colony growth on a weekly basis beginning on day 14. Precursor frequency was determined by the maximum likelihood method as described above. Medium was removed (100 uL) and cells fed every 2 weeks. Medium from colony positive wells was stored at -135°C for p24 assay.

B. Results

1. Interaction of HIV-1 and Bone Marrow.

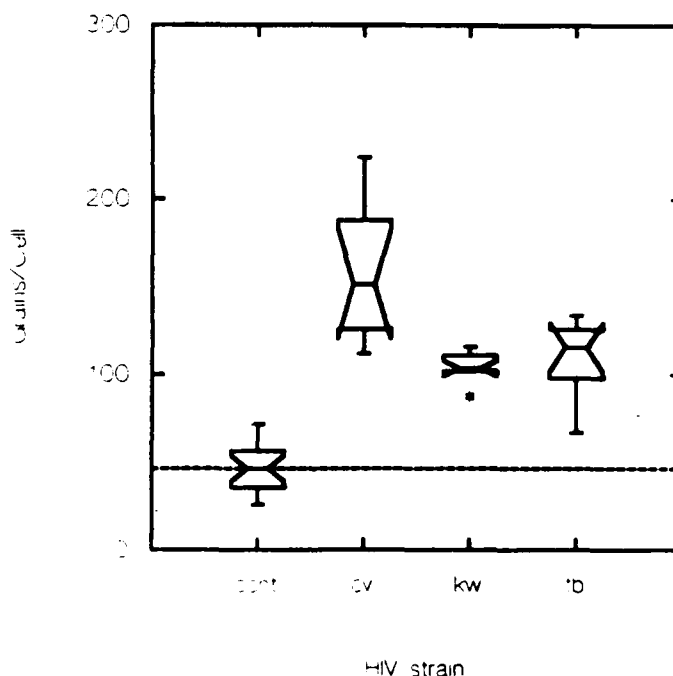
a. Culture of Human Bone Marrow Cells. Many of our planned experiments involve assessment of marrow growth and virus growth as a function of time after infection. This is difficult using currently available culture methods that use a semi-solid culture medium such as agar or methylcellulose. We have recently adapted a culture method described for the long term culture of monocytes. By using Teflon culture vessels and highly specific recombinant growth factors we are able to grow sufficient numbers of marrow cells and maintain them in culture for up to three months. Culture vessel and culture density aside, all other culture parameters are identical to that used in a conventional methylcellulose assay.

b. Lymphocyte and monocyte depleted bone marrow and purified marrow progenitor cells can be infected with HIV-1. We have used the system described above to grow human bone marrow cells that have been purified using two different methods. The object of these purifications is to remove cell types that are already known to be permissive to HIV infection. After lymphocytes and monocytes have been removed we can culture their progenitors and determine when during development

these cells become infectable. In this way we can determine whether HIV-1 growth is limited to mature peripheral blood cells or alternatively whether virus can be harbored in more primitive progenitor cells.

We have used Counterflow Centrifugal Elutriation and depletion with monoclonal antibodies plus complement (anti-CD4, anti LeuM1, anti-CD3) as a means of lymphocyte and monocyte depletion of bone marrow progenitors. We have infected lymphocyte/monocyte depleted human bone marrow with several strains of HIV and have used in situ hybridization, in collaboration with Dr. Opendra Narayan and his colleagues, to probe for expression of viral GAG sequences. We have detected viral GAG mRNA in the monocytoïd progeny of monocyte depleted bone marrow after 14 days culture in the presence of recombinant human M-CSF and GM-CSF, suggesting that cells earlier than those found in the periphery may serve as viral hosts (figure 1).

Figure 1. In situ hybridization for detection of HIV-1 GAG mRNA. Box plots show the distribution of grains in positive cells. Monocyte depleted BM was infected with 3 different clinical isolates of HIV-1 and processed after 14 days in culture. The majority of positive cells were morphologically identifiable as macrophages. A few cells of blast morphology also evidenced grains. Data are summarized in notched box plots which indicate median values (waist), simultaneous 95% confidence intervals about the medians (span of notch), interquartile range (bottom to top of box) and extreme values (bars) exclusive of outliers. Outliers are defined as data points more than 1.5 times the interquartile range beyond the top or bottom of the box and are plotted individually as asterisks.

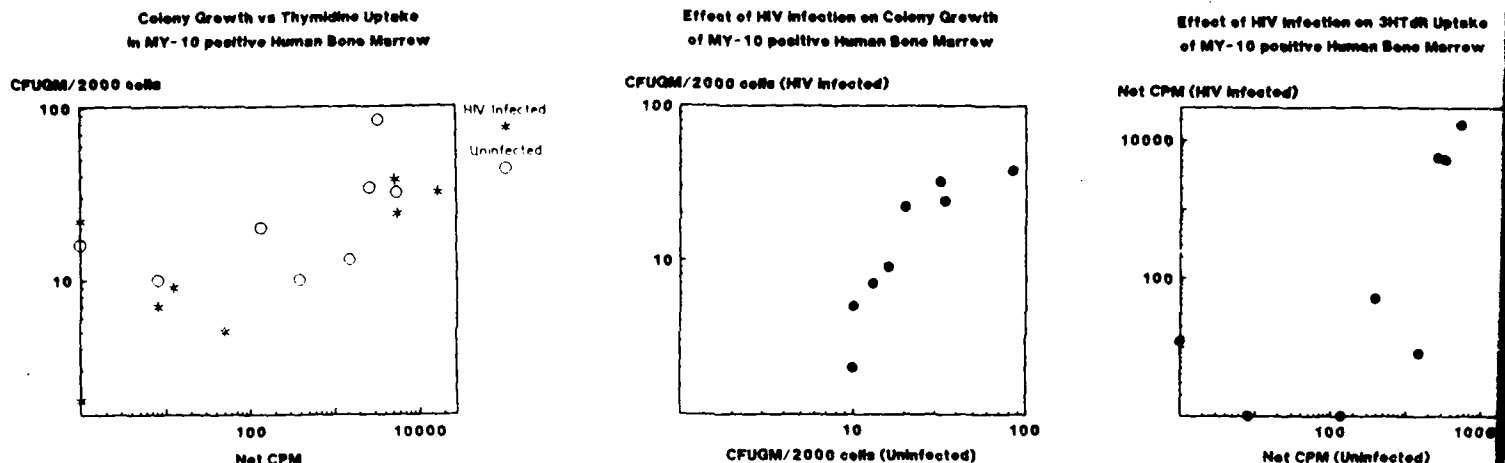


Although elutriated marrow is depleted of mature lymphocytes and monocytes it contains progenitor cells of all lineages at various stages of differentiation. We have taken advantage of a monoclonal antibody that identifies very early marrow progenitor cells (anti-CD34) to positively select a relatively homogeneous cell population for culture and infection. Studies involving progenitor growth at limiting dilution are now in progress (see below) and will determine whether these primitive multipotential marrow progenitors can be infected and transmit the virus to their progeny.

c. Effect of HIV-1 infection on growth of purified progenitor cells: comparison of conventional methylcellulose culture and Teflon liquid culture systems. We compared these two systems using visual colony count to assess progenitor growth in the methylcellulose system and tritiated

thymidine uptake to assess proliferation in the Teflon culture system. Purified marrow progenitor cells ($CD34^+$ [MY-10⁺] cells isolated by immune adherence) were used in both assays. Replicate cultures were established after 24 h adsorption or mock adsorption with HIV-1 strain KW (a monocyctotropic strain, the gift of Dr. H. Farzadegan). There was close correlation between the 2 assays (figure 2, A). Although a modest decrease in progenitor growth was detected by both colony count (figure 2, B) and thymidine uptake (figure 2, C), robust growth of both monocytoid and granulocytoid lineages were observed in the cultures derived from infected progenitors. The use of purified progenitor cells and recombinant exogenous growth factors suggest that reports describing severe inhibition of bone marrow colony growth by HIV-1 may reflect secondary effects of the virus on other cell populations present in unfractionated marrow (e.g. lymphocytes, monocytes).

Figure 2. Effect of HIV-1 infection on the growth of purified $CD34^+$ progenitor cells.



d. Limiting dilution analysis (LDA) of human bone marrow progenitor growth.

The results described above (section b.), as well as those reported recently by Folks et al (1989), in which monocyte/lymphocyte depleted bone marrow progenitors were shown to be infectable with HIV-1 (which was detectable in their monocytoid progeny 2 weeks later) are subject to a major criticism: that even low levels of contaminating infected monocytes (e.g. <0.1%) could harbor virus and transmit it to newly developing monocytes as they mature. We are now addressing this problem by using LDA, a quantitative method to determine the frequency with which cells having a specific functional property are present in a heterogeneous population. We have used this method extensively to lymphoproliferative responses (see below). Recently we have also adapted this methodology to the growth of human bone marrow progenitor cells; its advantage over bulk culture methods is that at limiting dilution one can determine with a given statistical precision that all cells present in a culture were derived from a single precursor. One can also determine the probability that a cell of a given phenotype (e.g. a lymphocyte or a monocyte) was also present in that culture. We are now using LDA to grow monocyte/lymphocyte depleted bone marrow (from HIV-1 infected subjects or marrow infected in vitro prior to culture). These studies should help resolve important question concerning the range of viral permissiveness in developing human bone marrow progenitor cells.

e. Development of antigen presenting cells from monocyte depleted bone marrow: a system to study the effects of HIV-1 infection on monocyte ontogeny. In order to study the effects of HIV-1 on the phenotypic and functional ontogeny of antigen presenting cells (APC), we have

developed a liquid culture system in which monocyte depleted bone marrow is driven to proliferate and differentiate with recombinant human GM-CSF and M-CSF in Teflon flasks. Ability of plastic adherent cells from fresh and cultured monocyte depleted bone marrow to present the protein antigen tetanus toxoid to autologous monocyte depleted lymphocytes was assessed by measuring $^3\text{HTdR}$ uptake after 5 days co-culture in microtiter wells. Generation of APC function and maximal CD4 expression (as measured in the monocyte scatter gate) required the presence of CSFs and a 14 day culture interval (table 1). In contrast, LeuM3⁺ phenotype developed after 7 days in culture with CSFs. This system provides a simple and rapid assessment of antigen presenting cell ontogeny, demonstrating the appearance of LeuM3 prior to detectable APC function.

Table 1. Development of Immunocompetent Monocyte/Macrophages from Cultured Monocyte Depleted Marrow: Antigen presenting capacity and Immunophenotype.

Day	Growth Factors	Antigen Pulse	$^3\text{HTdR}$ Uptake	% Positive LeuM3	CD4
0	No	No	314 (274, 424)	0.8	0.0
7	No	No	109 (52, 228)	5.4	28.0
7	Yes	Yes	150 (54, 498)	24.4	27.7
14	No	No	1953 (1734, 2192)	15.9	39.9
14	Yes	Yes	5336 (4428, 6430)	45.9	61.2

* Geometric mean CPM, parentheses: lower, upper 95% confidence intervals.

In two independent experiments, monocyte depleted bone marrow was inoculated with HIV-1 (lymphotropic HTLV-III_B or monocyto-tropic SF162 strains), washed after 24 h, cultured with CSFs for 14 days, and transferred to microtiter wells. Cells adhering to the plastic wells were then irradiated and tested for the ability to present tetanus toxoid antigen by co-culture with autologous monocyte depleted lymphocytes. Antigen specific lymphoproliferation was increased 2 to 3-fold over that observed when mock infected adherent cells were used as APC. Intimate contact between APC and lymphocytes during immune interactions may be a mechanism by which the virus facilitates its own transmission and propagation. It is not presently known whether the observed increase in thymidine uptake is due directly to interaction of lymphocytes with APCs that are actively expressing HIV gene products (approximately 10%).

Table 2. Ability of HIV-1 Infected and Uninfected Adherent Cells Derived from Adherence Depleted Cultured Marrow to Present Tetanus Antigen to Autologous Monocyte Depleted Lymphocytes

BM Treatment		Net CPM Mean (SEM)
M+GMCSF	HIV IIIB	
No	No	1,358 (309)
No	Yes	3,444 (634)
Yes	No	2,946 (836)
Yes	Yes	10,507 (617)
No APC		1,360 (32)
No APC, No TET		196 (49)

f. Bone Marrow Transplantation in AIDS. One of the stated AIMS of this contract is to model the use of BMT as a possible treatment for AIDS. This area has taken a leap forward with the first transplant of an HIV-1 seropositive patient using full myeloablation and azidothymidine. This laboratory has collaborated in this project by monitoring immune reconstitution after transplantation. The patient, who in addition to his HIV disease presented with an aggressive lymphoma, died of relapse of his tumor 50 days after transplant. Before his death, several important parameters of antigen specific immunity had been restored. At autopsy we were unable to detect virus using polymerase chain reaction gene amplification, the most sensitive technique available. We have a current protocol to transplant more HIV-1 positive patients who have hematologic malignancies and are exploring the possibility of initiating a protocol to transplant a carefully selected population of HIV-1 seropositive patients who do not have cancer. These results have been published in abstract form (V International AIDS conference, Montreal) and are currently in press as a full manuscript (Annals of Internal Med).

2. Evaluation of immunologic changes related to progression of HIV infection.

a. Limiting Dilution Analysis of In Vivo Activated (IL-2 Responsive) Peripheral Blood Lymphocytes in HIV-1 Infected Subjects. Using conventional bulk culture of peripheral blood mononuclear cells (PBMC), we confirmed the results of others that HIV infected patients manifest a loss of T cells that can respond to IL-2. Thus PBMC from AIDS patients respond more poorly than those from patients with AIDS related complex (ARC), which respond more poorly than cells from asymptomatic HIV-infected subjects; all HIV-infected groups have weaker responses than uninfected people. Using a novel highly quantitative assay to estimate the proportion of IL-2 responsive cells and examine responsiveness on a "per cell" basis, we found in a cross-sectional study of a spectrum of HIV-infected homosexual men that there was a progressive decrease in the number of IL-2 responsive cells decreases as the disease progressed. However, a potentially very important finding was that individual IL-2 responsive cells from asymptomatic infected persons or ARC patients responded as well as individual IL-2 responsive cells from healthy noninfected persons. In contrast, AIDS patients not only had fewer IL-2 responsive cells, but those that did respond gave rise to fewer progeny cells. These results suggest that an impairment in responsiveness to IL-2 may limit the ability of AIDS patients to amplify responding immune cells. A manuscript describing our findings has been published in Clinical Immunology and Immunopathology.

To confirm and extend these results, and to evaluate rigorously the value of measurement of IL-2 responsive lymphocytes for staging and prognostication of HIV infection, a prospective study is needed. We have recently initiated such a study using PBMC from seroconverters in the Baltimore chapter of the Multicenter AIDS Cohort study. This study will be supervised by Dr. Rajesh Chopra who is joining our laboratory as a Research Associate with the support of this contract. We plan to study as many

seroconverters as possible at six-month intervals to determine whether changes in number and function of IL-2 responsive cells add to the power of the prognostic information already available, such as CD4 cell number and serum p24 level. We will also study HIV-seronegative cohort members in parallel to provide a control for interpretation of data. To date two pairs of seroconverters with concurrent seronegatives have been studied, and data are pending analysis.

b. **Changes in T cell subsets.** Analyzing data acquired over the first three years of the multicenter AIDS cohort study (MACS), we demonstrated that when CD3⁺, CD4⁺, and CD8⁺ lymphocytes are determined independently (one-parameter analyses), an increase in CD3 - (CD4 + CD8) occurs at the time of seroconversion and continues afterward. This increase is present in both absolute and relative terms and cannot be accounted for by changes in the CD4⁺ and CD8⁺ populations of T cells. The most likely explanations for these findings were that either 1) there is an increase in "double negative" T cells, i.e., lymphocytes expressing the CD3⁺CD4⁻CD8⁻ phenotype, or 2) there is a decrease in natural killer (NK) cells, some of which express the CD3⁺CD4⁻CD8⁺ phenotype. A paper describing these findings and discussing these possible explanations for the findings has been published recently (Margolick et. al., Clin. Immunol. Immunopathol. 57:348-361, 1989).

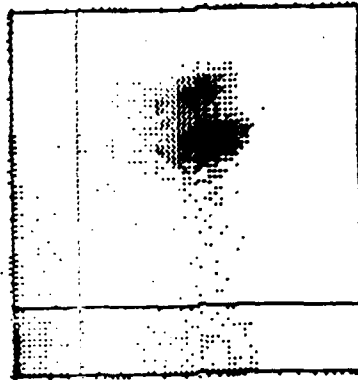
To test these hypothetical explanations for the increase in calculated double negative cells present in seropositive individuals compared to seronegatives, we are studying seropositive and seronegative subjects participating in SHARE (homosexual men) and ALIVE (a cohort of intravenous drug users also being followed prospectively at Johns Hopkins beginning in February, 1988). Preliminary results derived from the 65 patients studied to date (34 seronegative for HIV-1 and 31 seropositive, including 4 with AIDS) are shown in table 3. It is important to note in this table that the expected increase in absolute number of calculated double negative cells was indeed observed and was statistically significant. This result permits a more detailed analysis of the relative contributions of the two cell populations which we expect to account for this increase.

Table 3. Absolute numbers (per mm³) of lymphocytes in seronegative (SN) and seropositive (SP) homosexual men.

	SN	SP	P
Lymphocytes	2387 ± 503 (33)	1928 ± 755 (30)	0.006
Calculated	26.1 ± 57.0 (33)	95.8 ± 74.1 (29)	<0.001
Double Neg.			
Measured	68.2 ± 41.2 (33)	90.1 ± 50.1 (29)	0.064
Double Neg.			
γδ-TCR	59.5 ± 39.9 (33)	86.5 ± 63.0 (29)	0.046
Dim CD8	153.7 ± 68.7 (20)	112.7 ± 63.8 (22)	0.052
NK	227.1 ± 174.4 (10)	79.1 ± 55.0 (10)	0.048

To evaluate the possibility of an increase in true (as opposed to calculated) double negative T cells, we have stained lymphocytes with antibodies to CD3 (fluorescein-conjugated) and a mixture of CD4 and CD8 (phycoerythrin-conjugated) in a single specimen, so that lymphocytes with the CD3⁺CD4⁻CD8⁻ phenotype can be measured directly and unambiguously (figure 3). In this figure, the double negative lymphocytes are those in quadrant 4 (lower right quadrant) which fluoresce positively with anti-CD3 (green) but not anti-CD4 or anti-CD8 (red). We hypothesize that these cells may represent a T cell precursor population.

Figure 3. Use of 2 color immunofluorescent staining to detect CD3 positive CD4⁻, CD8⁻ T cells (lower right quadrant). These cells represent 3% of the total lymphocytes.



As shown in Table 3, the absolute number (cells/mm³) of lymphocytes with the double negative (CD3⁺CD4⁻CD8⁻) phenotype were significantly increased in seropositive individuals as compared to seronegatives. A similar increase was also present in relative (percent) terms (not shown). Most of this change appeared to be due to an increase in T cells (CD3⁺) expressing the gamma/delta-T cell receptor rather than the more common alpha/beta-receptor, as indicated by significant increases in the absolute number and proportion (not shown) of lymphocytes staining with an antibody (TCR δ -1, T Cell Sciences, Cambridge, MA) to the delta chain of the T cell antigen receptor.

To evaluate the possibility of a fall in NK cells expressing the CD3⁺CD4⁻CD8⁺ phenotype, we have taken advantage of the fact that some NK cells express CD8 at low levels. Therefore, we have measured the proportion and number of dim CD8⁺ lymphocytes. Although the proportion of such cells was not decreased in seropositive individuals (not shown), the absolute number was significantly decreased (Table 3), consistent with this possibility. In further support of this interpretation, there was a large (67%) decrease in the absolute number of lymphocytes expressing the Leu19⁺CD3⁺ phenotype characteristic of NK cells in seropositive as compared to seronegative subjects. This decrease was statistically significant despite the smaller number of subjects studied.

Taken together, these results suggest that the increase in calculated CD3 minus (CD4 plus CD8) is due to both an increase in double negative T cells and a decrease in natural killer cells. On the basis of these preliminary data, the contribution of the decline in NK cells appears to be about twice that of the increase in true double negative T cells. A role for NK cells in host defense against HIV has been postulated, but most studies have not found a decrease in NK cell numbers until after the onset of AIDS. Our results suggest that this decline occurs much earlier than has been thought, and it will therefore be important to determine if this decrease is of prognostic significance. Studies to investigate this possibility using the MACS data are underway. In addition, our data are consistent with the hypothesis that double negative T cells, as well as T cells expressing the $\gamma\delta$ -TCR, may play a role in defense against HIV.

These results have been submitted to the annual meeting of the Clinical Immunology Society.

c. **Magnitude of antigen- and mitogen-induced lymphoproliferation.** To determine whether loss of antigen-induced T cell proliferative responses are predictive of, or merely coincident with disease progression, we are now following these responses among SHARE seroconverters. This population has been chosen because they are still in the early stages of decline and therefore can provide baseline data against which subsequent values may be compared. Such a foundation is needed in view of the notorious variability of lymphoproliferative responses among individuals and between individuals, especially with respect to antigens. We are also evaluating responses to T cell dependent mitogens (PHA, PWM, Con A) both as positive controls for the soluble protein antigens and to evaluate the

prognostic value of these parameters as well. Another strength of the experimental design is that a seronegative participant is studied concurrently with each seroconverter, in order to provide a positive control for reagents and cell preparation. To date 19 seronegatives and 20 seroconverters have been studied on one occasion each; so no data on correlation with disease progression are available yet. More patients are being enrolled in the study each week. These patients will be evaluated at all subsequent semi-annual visits to the clinic.

d. Expression of HLA-DR by monocytes of HIV-seropositive individuals. To test the possibility that decreases in HLA-DR expression which lie within the normal range may contribute to immune suppression in HIV infection, we are prospectively evaluating expression of HLA-DR by peripheral blood monocytes of homosexual men who are seropositive for HIV. This study is being conducted prospectively in participants in SHARE, the Baltimore chapter of MACS. We are evaluating subjects who have developed antibodies during the study, so that the time of infection with HIV is known to within four months. Seronegative and long-term seropositive subjects are also studied for purposes of quality control and establishing normal levels of expression of HLA-DR by monocytes. For these studies, the monocyte population is defined by light scatter characteristics and by positive staining with the monoclonal antibody anti-LeuM3 which stains most monocytes but not lymphocytes.

To date, we have studied monocytes from 72 seroconverters. All but one of these have had normal percentages of monocytes expressing HLA-DR, i.e., more than 85% of monocytes positive for HLA-DR. These levels have not changed when these subjects have returned for follow-up evaluations, at least during the first such follow-up. We are also measuring intensity of HLA-DR expression on a per cell basis, so that not only number of positive monocytes but degree of positivity can be factored into this analysis in the future. These studies have been delayed somewhat by a subtle hardware problem in the flow cytometer which required re-calibration of the instrument this year, but we are now following the patients as they return for their regular clinic evaluations.

e. Analysis of cerebrospinal fluid from HIV-seropositive individuals. One of the special research foci of the Multicenter AIDS Cohort study is a prospective evaluation of risk factors for development and progression of neurological disease in individuals infected with HIV. In collaboration with Dr. Justin McArthur of the Department of Neurology, Johns Hopkins Hospital, we have developed a method for phenotypic analysis of lymphocytes in cerebrospinal fluid (CSF) by 2-color flow cytometry (Margolick et al, 1988). We have found that in all stages of HIV-associated neuropathology, as well as in control subjects without neurologic disease, the proportions of CD3+, CD4+, and CD8+ lymphocytes in the CSF are virtually the same as those in the peripheral blood (Margolick et al, 1988). This result suggests the absence of preferential recruitment of lymphocyte subtypes to the CSF, and the lack of preferential T cell proliferation in the CSF. We are now expanding our analysis to address the question of whether there is an increased proportion of activated T cells among the lymphocytes in the CSF in HIV-seropositive homosexual men. To accomplish this goal, we are capitalizing on our capability of double-staining these cells, and using the lymphocyte activation markers CD38 and HLA-DR in combination with CD3, CD4, and CD8 as previously done. To date, CSF and peripheral blood from 3 subjects have been studied. The analysis is still underway, but preliminary results indicate that the proportions of activated CD4+ and CD8+ T lymphocytes are again virtually identical in the blood and CSF. If this result holds up, it will suggest that monitoring of these lymphocyte markers in the CSF will not be prognostically useful, and that other markers should be investigated to identify lymphocyte changes in the CSF that may indicate local HIV-associated disease.

3. Discussion related to goals.

a. Original Goals. This contract proposal was submitted under the Broad Agency Announcement and underwent two cycles of review prior to funding (original submission date February, 1986; funded December, 1987). In response to the requests of Drs. Bancroft and Noyes two major revisions were made to the goals as originally submitted: 1) studies specifically dealing with potential autoimmune mechanisms of HIV-1 pathogenesis were deemed not to be of sufficient interest and were therefore omitted, 2) studies on the in vitro effects of the drug Cyclosporine A on HIV-1 growth were deemphasized on the basis of newly available data indicating that Cyclosporine A had no apparent clinical role in the management of HIV-1 infection.

b. **Amended Goals.** We have amended our research goals in keeping with progress in the field and the unique resources available to us through our involvement in the Johns Hopkins Marrow Transplantation Program and Hopkins-based cohort studies of the natural history of HIV infection. These revised goals are faithful to the original intent of the proposal in that they are directed toward an understanding of interactions of HIV-1 and the hematopoietic system and toward the establishment of immunologic markers of disease progression. These goals were discussed in detail with our project officer, Dr. Peter Gomas, at our site visit of April, 1989.

VII. CONCLUSIONS

A. Summary of implications

1. **Newly adapted marrow culture techniques provide improved experimental capabilities.** The adaptation of the Teflon culture system to bone marrow growth has provided a means to obtain quantitative recovery of cells of the monocyte macrophage lineage that have differentiated in bone marrow cultures. These were difficult to recover using other methods owing to their adherent properties. The adaptation of a quantitative assay of precursor growth by limiting dilution analysis has provided a powerful method to assess cloning efficiency and to isolate cells that are the progeny of a single progenitor cell.

2. **Development of an inexpensive p24 assay.** We have determined optimal conditions for a p24 antigen capture assay using widely available inexpensive reagents. Although sensitive and specific assay kits are commercially available, these cost up to \$20 per individual test. Our assay compares favorably in sensitivity with the Abbott test and costs approximately \$7 per 96 replicates.

3. **Bone marrow progenitor cells can be infected with HIV-1.** Our early results indicate that monocytes arising from cultured monocyte/lymphocyte depleted bone marrow express HIV-1 mRNA. These data suggest that a cell developmentally earlier than an identifiable monocyte was infected at the initiation of culture and that viral expression occurred concomitantly to differentiation. These results must be interpreted with caution, however, since contamination of the initial cell inoculum with very small numbers of monocytes (and subsequent spread to newly differentiated cells) could potentially explain these results.

4. **Data collected thus far show little effect of HIV-1 infection on progenitor cell growth and function of progeny.** Colony forming efficiency and proliferative capacity of purified (CD34⁺) progenitor cells were only mildly reduced when progenitor cells were incubated with virus prior to culture. Mature monocytes derived from infected progenitor cells were able to present antigen to autologous monocyte depleted lymphocytes.

5. **Adoptive transfer of donor immunity and elimination of detectable virus in the first bone marrow transplantation for HIV-1-associated hematologic malignancy.** Although the patient died soon after transplant of a lymphoma relapse, virus was undetectable by culture or polymerase chain reaction gene amplification. Lymphoproliferative and antibody responses to recall antigens that had been ablated by HIV disease were reconstituted. We will monitor the immune status of other HIV⁺ patients transplanted under this protocol.

6. **IL-2 responsive cells decline in number and individual proliferative capacity with progression of HIV infection.** This finding needs to be confirmed by prospective studies, but it suggests that understanding the mechanism of this decline may offer new insights into prognostication of disease progression and therapy of the infection. Of particular importance will be the identification of the cell types involved (T cells versus B or LAK cells) and the mechanism of the decreased proliferative capacity of individual IL-2 responsive cells seen in AIDS but not other stages of HIV infection.

7. **HIV-1 infection is associated with changes in minor T cell subsets.** Specifically, our data indicate that there is an increase of approximately 50% in CD3⁺CD4⁺CD8⁻ lymphocytes and as much as a 67% decline in natural killer cells in seropositive individuals as compared to seronegatives. The

mechanisms and biologic importance of these changes remain to be determined, but the possibility of useful insights into the pathogenesis of HIV infection merits investigation.

8. HLA-DR expression appears to be within normal limits on monocytes from HIV-1 infected individuals. Although the possibility of changes within individuals over time has not been ruled out, this important component of the immune system appears to be relatively unaffected by HIV infection.

9. The proportion of activated (CD38⁺ or HLA-DR⁺) T lymphocytes in the blood and cerebrospinal fluid of HIV-infected homosexual men are indistinguishable. These are very preliminary data and are based on the 3 patients studied to date.

B. Recommended future directions: plans for the next quarter.

1. Interaction of HIV-1 and Bone Marrow.

a. Bone Marrow Transplantation. The Johns Hopkins Bone Marrow Transplant Unit will continue to recruit appropriate HIV-1 and HTLV-1 seropositive patients with hematologic malignancies for combined modality (azidothymidine plus marrow ablation) allogeneic bone marrow transplantation. An expanded protocol for HIV patients without malignancies is under consideration, pending identification of the appropriate poor prognosis population. We will continue to monitor humoral and cell mediated immunity in these patients, and develop strategies for adoptive immunotherapy through immunization of both graft donor and recipient.

b. Experimental infection of progenitor cells. We will continue our studies of experimental HIV-1 infection of enriched populations of bone marrow progenitor cells. Limiting dilution analysis will be used to look for viral expression in the progeny of individual progenitor cells. We will also initiate studies in progenitor cells isolated from the bone marrow of HIV⁺ subjects.

2. Immunologic Markers of Disease Progression.

We will continue the prospective immunologic studies described above; emphasis will continue to be on individuals who have seroconverted at known times, so that changes in lymphocyte and monocyte phenotype can be assessed in the context of the natural history of HIV infection.

a. HLA-DR expression. We will continue to monitor expression of Class II histocompatibility antigens (HLA-DR) on monocytes from homosexual men with and without HIV infection.

b. Double negative T cells. We will continue to measure numbers and percentages of lymphocytes which express the characteristics discussed above, including 1) the $\gamma\delta$ -T cell receptor; 2) the CD3⁺CD4⁻CD8⁻ (double negative) phenotype; and 3) the Leu19⁺CD3⁺ phenotype characteristic of natural killer cells.

c. In vivo activated IL-2 responsive lymphocytes. We will continue to measure IL-2 responsive cells in patients at various stages of HIV infection to determine whether this measurement can help identify new stages of disease progression.

d. Use of cryopreserved peripheral blood cells from prospective cohort studies. We will evaluate whether frozen cells, available on most subjects before and after seroconversion, can be used for the experiments described in a, b, and c. This would have the great advantage of permitting nested case-control studies to test the hypothesis that these measurements have prognostic and/or pathogenetic significance. Additionally, such analyses could be performed in a relatively short time compared to newly initiated prospective studies.

e. Biology of the limitation of T cell proliferation in AIDS. We will initiate functional studies of activated T cells in the peripheral blood of HIV infected individuals. These studies will be conducted by Dr. Rajesh Chopra, who will join our group by October 1, 1989. These studies will investigate the number and function of in vivo activated T lymphocytes in seroconverters and other patients, as judged by the ability of T cells to proliferate in limiting dilution assays in the presence of interleukin 2 (IL-

2; T cell growth factor) and no other stimulus for growth. These studies will determine the mechanisms of the defect and the specific types of lymphocytes affected.

f. **Analysis of cerebrospinal fluid from HIV-seropositive individuals.** We will continue to analyze the co-expression of activated (HLA-DR+ and CD38 along with CD4 and CD8 on CSF lymphocytes. If no differences are found between expression in CSF and expression in peripheral blood, other activation markers will be investigated.

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